Pre-Summer Preparation work

- Was introduced to the Bowman-Birk Inhibitor(BBI) by Dr. Aaron Goodarzi, who is a radiooncology researcher at the Charbonneau Cancer Institute at the University of Calgary
    - BBI: a protease inhibitor derived from soybeans, which was found to have radioprotective properties by a lab in Germany headed by Dr. Klaus Dittman (Dittmann K, Loffler H, Bamberg M, Rodemann HP. Bowman-Birk proteinase inhibitor (BBI) modulates radiosensitivity and radiation-induced differentiation of human fibroblasts in culture. Radiother. Oncol. 1995;34:137–143.)
- Decided on doing the BBI as our radioprotective peptide of choice; found the sequence of the truncated or modified version of the BBI which has been shown to retain its functions as a radioprotector (Dittmann KH, Gueven N, Mayer C, Rodemann HP. Characterization of the amino acids essential for the photo- and radioprotective effects of a Bowman-Birk protease inhibitor-derived nonapeptide. Protein Engineering. 2001;14:157–160.)
    - The whole BBI protein (70 a.a.) can cause problems in blood clotting; 9 a.a. Sequence avoids that issue, because the section of the peptide that acts as a protease inhibitor is cut off
- Previous literature has also suggested a potential molecular pathway by which BBI works. (Gueven N, Dittmann K, Mayer C, Rodemann HP. Bowman-Birk protease inhibitor reduces the radiation-induced activation of the EGF receptor and induces tyrosine phosphatase activity. Intl. J. Radiat. Biol. 1998;73:157–162.) BBI mostly works in Non-Homologous End Joining pathways, which is expected as that is the most prevalent mode of DNA double stranded repair in cells
- Looked into other potential peptides that we can use in case the BBI does not work out: KTI, Glutathione, antioxidants
    - KTI: another radioprotective peptide that has been found to be effective in protecting against certain types of radiation that the BBI does not (Van den Hout, R.; Pouw, M.; Gruppen, H. Inactivation kinetics study of the Kunitz soybean trypsin inhibitor and the Bowman−Birk inhibitor. J. Agric. Food Chem. 1998, 46 (1), 281−285.)
    - Glutathione: a reactive oxygen species “sink” that quenches reactive oxygen species by binding to its lone electron. Mostly works near the mitochondria of the (Chatterjee A. Reduced Glutathione: A Radioprotector or a Modulator of DNA-Repair Activity? *Nutrients*. 2013;5(2):525-542. doi:10.3390/nu5020525.)
    - Antioxidants: works by a variety of pathways (Weisse J. F. and Landauer M. R., Radioprotection by Antioxidants. *Annals of the New York Academy of Sciences.* 2000; 899: 44–60. doi:10.1111/j.1749-6632.2000.tb06175.x)
- Also looked at current other methods of radioprotection (doubles as market research for the application of our device) (Kamran, M. Z., Ranjan, A., Kaur, N., Sur, S. and Tandon, V. (2016), Radioprotective Agents: Strategies and Translational Advances. Med. Res. Rev., 36: 461–493. doi:10.1002/med.21386)
    - Currently some radioprotectors have been proposed for cancer radiotherapy (ex. synthetic thiol-containing compounds, amifostine) but uptake by the public and medical community has not been great as side-effects were undesirable and drug benefits were not well defined
    - NASA Biocapsule - we did not know about this the time when we came up with our project, but after some searching around we stumbled apon this on the internet. Comparable technology to our device, unsure about the progress/research that has been done on this as NASA is very secretive (http://sservi.nasa.gov/articles/nasa-breakthrough-could-save-millions-lives/; http://www.medgadget.com/2012/02/nasa-biocapsule-implant-diagnoses-and-treats-diseases-without-human-intervention.html)
- Were unsure about the specific potential application of our peptide: it was a debate between cancer radiation prevention (as radiotherapy is known to cause secondary

cancers, and certain existing literature stated that BBI has radioprotective effects only on p53+ cells, which about 60% of all cancers are p53-) (Dittmann KH, Gueven N, Mayer C, Ohneseit P, Zell R, Begg AC, Rodemann HP. The presence of wild-type TP53 is necessary for the radioprotective effect of the Bowman-Birk proteinase inhibitor in normal fibroblasts. Radiation Research. 1998;150:648–655.), or space application
    - We realize these applications would call for very different designs for our device as well as different methods of testing, but those differences exist mostly in the more refined details of our project, which would not affect our preliminary work
    - Decided that we needed a deadline by which to decide the application of our project: End of June (tentative)
- Planned out some experiments we wanted to do to test viability of the various peptides: Clonogenic survival assay with both cancer cells as well as primary cells
    - We had a few variations on the clonogenic survival: thought about doing high throughput assays with 96 well plates, dual plating with cancer and primary cells to see interactions, comparison of survivability of cancers vs. primary cells with and without BBI treatment
- Expected results: BBI is expected to protect our primary cells against radiation while not protecting our cancer cells as much as the primary cells. Meaning that the surviving fractions of our cell lines treated with BBI will be higher than that of cell lines not treated with BBI, and the surviving fractions of primary cell lines treated with BBI will be higher than that of cancer cell lines treated with BBI

May 3, 2016

Dr. Goodarzi Interview

- We met with Dr. Goodarzi to talk about potential assays and our prospects for the summer
- Dr. Goodarzi confirmed the viability of our clonogenic survival assay in testing the effectiveness of BBI and mBBI, but he also suggested a few more assays we can use, namingly the H2AX assay (Mariotti LG, Pirovano G, Savage KI, Ghita M, Ottolenghi A, Prise KM, et al. (2013) Use of the γ-H2AX Assay to Investigate DNA Repair Dynamics Following Multiple Radiation Exposures. PLoS ONE 8(11): e79541. doi:10.1371/journal.pone.0079541) and the Flow cytometry assay
    - H2AX: Can use fluorescent tags to tag H2AX foci (histone proteins that localize around double stranded breaks) to detect the number of double stranded breaks post irradiation with or without BBI. Using fluorescent microscopy to view double stranded breaks post irradiation, we can blind count the number of detected foci at various timepoints within 24 hours of irradiation to see if the cells treated with BBI are able to repair their double stranded breaks (marked by the decrease of fluorescent foci) at a faster rate comared to cells that are not
- Dr. Goodarzi had also suggested that we use non-cancer cell lines as well as cancerous cell lines to test the difference in radiation protection in cancer cells versus non-cancerous cell lines
- Listed a number of cell lines that will be able to provided for us, including a variety of cancerous cell lines as well as normal cell lines

May 4, 2016

Laboratory introduction and techniques week

- Made and Autoclaved agar (1L)
    - 1% tryptone (10g)
    - 0.5% Yeast extract (5g)
    - 1% NaCl (10g)
    - 1.5% Agarose (15g)
- Poured plates of LB agar with 3 different types of antibiotics:

- Amp (stock 100 mg/mL; final 100 ug/mL)
- Kan (stock 50 mg/mL; final 50 ug/mL)
- Chlor (stock 50 mg/mL; final 30 ug/mL)

May 5, 2016

Laboratory introduction and techniques week

- Results: Cultured competent E.Coli as well as Chlor-resistant E.Coli on each type of Agar plate(s)
  - Growth of a lawn of red-coloured bacteria (Chlor-resistant culture on Chlor plate & Chlor-resistant culture on Amp plate)
  - Growth of a lawn of white/yellow- coloured bacteria (competent E.Coli culture on Amp plate) --> contamination (?)
  - No growth observed on other plates
- Results: Liquid Culture of normal as well as Chlor-resistant E.Coli (no antibiotic added to liquid culture)
  - Growth of white/yellow bacterial cultures on the bottom of all tubes
- Made 50mM $CaCl_2$ (5mL of 1M $CaCl_2$, 95mL ddH_2O) & 50mM $CaCl_2$ with 15% Glycerol (5mL 1M $CaCl_2$, 15mL Glycerol, 80mL ddH_2O) for making competent E.Coli --> placed in 4°C fridge
- Inoculated 2 tubes of 5 mL LB with old (#10) competent *E. coli* cells
- Inoculated 2 tubes of 5 mL LB with new (C3037) competent *E. coli* cells (Used steril 5 mL LB as negative control) --> incubated at 37°C O/N, shaking (200 rpm)

May 6, 2016

Laboratory introduction and techniques week

- The overnight cultures of competent *E. Coli* (#10 and C3037) were subcultured in 49ml of LB. There were four subcultures total: 2xC3037 and 2x#10. Cultures were incubated at 37.5°C and shaken at 200rpm for 4 hours. The cultures were grown to an optical density of 0.647 (within the range of 0.4-0.6)
- Chemical comptenece
  - Cultures were spun down for 7 minutes at 3000rpm and 4°C
  - 12.5ml of cold 50mM $CaCl_2$ was added and the pellet resuspended
  - Cells were then incubated on ice for 10 minutes
  - Cells were spun down again at 1500rpm for 2 minutes at 4°C
  - Cells were resuspended in 2ml of 50mM $CaCl_2$with 15% glycerol
  - Transfer to microcentrifuge tube in 100µl aliquots
  - Storage at -80°C
- The rest was left up to chassis team to document and discuss results

May 9, 2016: EXPERIMENTAL GOALS FOR THE SUMMER - DISCUSSION

BBI Efficiency
- test in human cell lines
- requires positive/negative control

Cytotoxic treatment
- induce DNA damage from something other than radiation/determination of mechanism
- H2AX assay

Circuit Function
- testing construct components
- promoters and RBS
- test with reporter genes

- BBI expression
- use mass spec/MALDI to measure peptide amount
- use rtQ-PCR as a backup

Excretion (overlap with Chassis)

- similar assay to BBI excretion

May 10, 2016

- Researched the demand for a radioprotective strategy in space exploration, found that NASA and the CSA are trying to further research efforts in this area, making it an important issue to address (http://www.space.com/21353-space-radiation-mars-mission-threat.html)
- Similar products are currently under development by NASA called the biocapsule (http://www.americaspace.com/?p=13700)
  - The developments of the biocapsule technology is unknown in the public, so we do not know how similar the biocapsule is from our proposed project

May 11, 2016

Peptide Sequences

- Compiled the mBBI amino acid sequences found in literature searches, and also put in some of our own additions to prime the peptide for secretion and delivery, as well as to accommodate our own assays
- Reverse translated them into genetic sequences for E.Coli as well as B. Subtilis

Common Identifier	FASTA	E. Coli	B. Subtilis	D. Radiodurans
(S) 9mer	CALSYPAQC	TGC-GCG-CTG-AGC-TAT-CCG-GCG-CAG-TGC	TGC-GCA-CTG-TCA-TAT-CCG-GCA-CAA-TGC	UGC-GCC-CUG-AGC-UAC-CCC-GCC-CAG-UGC
(V) 9mer	CALVYPAQC	TGC-GCG-CTG-GTG-TAT-CCG-GCG-CAG-TGC	TGC-GCA-CTG-GTT-TAT-CCG-GCA-CAA-TGC	UGC-GCC-CUG-GUG-UAC-CCC-GCC-CAG-UGC
(S) 7mer	ALSYPAQ	GCG-CTG-AGC-TAT-CCG-GCG-CAG-TGC	TGC-GCA-CTG-TCA-TAT-CCG-GCA-CAA-TGC	GCC-CUG-AGC-UAC-CCC-GCC-CAG
BBI protein	SACKSCICALSYPAQCFCVDIT	UCC-GCG-UGC-AAA-UCC-UGC-AUU-UGC-GCG-CUG-UCC-UAU-CCG-GCG-CAG-UGC-UUU-UGC-GUG-GAU	TCA-GCA-TGC-AAA-TCA-TGC-ATT-TGC-GCA-CTG-TCA-TAT-CCG-GCA-CAA-TGC-TTT-TGC-GTT-GAT-ATT-ACA	
Peptide #1 BBI	KSCICALSYPAQCF	AAA-AGC-TGC-ATT-TGC-GCG-CTG-AGC-TAT-CCG-GCG-CAG-TGC-TTT	AAA-TCA-TGC-ATT-TGC-GCA-CTG-TCA-TAT-CCG-GCA-CAA-TGC-TTT	AAG-AGC-UGC-AUC-UGC-GCC-CUG-AGC-UAC-CCC-GCC-CAG-UGC-UUC
Peptide #2 - NLS	GPKKKRKVKSCICALSYPAQCF	GGC-CCG-AAA-AAA-AAA-CGC-AAA-GTG-AAA-AGC-TGC-ATT-TGC-GCG-CTG-AGC-TAT-CCG-GCG-CAG-TGC-TTT	GGC-CCG-AAA-AAA-AAA-AGA-AAA-GTT-AAA-TCA-TGC-ATT-TGC-GCA-CTG-TCA-TAT-CCG-GCA-CAA-TGC-TTT	GGC-CCC-AAG-AAG-AAG-CGC-AAG-GUG-AAG-AGC-UGC-AUC-UGC-GCC-CUG-AGC-UAC-CCC-GCC-CAG-UGC-UUC
Peptide #3	KSCICALSYPAQCF	AAA-AGC-TGC-ATT-TGC-GCG-CTG-AGC-TAT-CCG-GCG-CAG-TGC-TTT-T	AAA-TCA-TGC-ATT-TGC-GCA-CTG-TCA-TAT-CC	AAG-AGC-UGC-AUC-UGC-GCC-CUG-AGC-UAC-CCC-GCC-CAG-UGC-UUC-UA

BBI HA	YPYDVPDYA	AT-CCG-TAT-GAT-GTG-CCG-GAT-TAT-GCG	G-GCA-CAA-TGC-TTT-TAT-CCG-TAT-GAT-GTT-CCG-GAT-TAT-GCA	C-CCC-UAC-GAC-GUG-CCC-GAC-UAC-GCC
Peptide #4 BBI NLS-HA	GPKKKRKVKSCICALSYPAQCFYPYDVPDYA	GGC-CCG-AAA-AAA-AAA-CGC-AAA-GTG-AAA-AGC-TGC-ATT-TGC-GCG-CTG-AGC-TAT-CCG-GCG-CAG-TGC-TTT-TAT-CCG-TAT-GAT-GTG-CCG-GAT-TAT-GCG	GGC-CCG-AAA-AAA-AAA-AGA-AAA-GTT-AAA-TCA-TGC-ATT-TGC-GCA-CTG-TCA-TAT-CCG-GCA-CAA-TGC-TTT-TAT-CCG-TAT-GAT-GTT-CCG-GAT-TAT-GCA	GGC-CCC-AAG-AAG-AAG-CGC-AAG-GUG-AAG-AGC-UGC-AUC-UGC-GCC-CUG-AGC-UAC-CCC-GCC-CAG-UGC-UUC-UAC-CCC-UAC-GAC-GUG-CCC-GAC-UAC-GCC

May 12, 2016

- Asked for peptide quotes from a multitude of peptide synthesis companies to find the cheapest and best synthesis deal

May 14-15, 2016: Lethbridge Workshop

Imagine, Design, Create – Cesar Rodriguez (cesar,.rodriguez@med.fsu.edu)

- imagine: Write it down!
    - Cell based therapeutics is the third pillar of medical therapeutics (small molecule → proteins/antibiotics → cells)
    - If[molecule]>x THEM produce therapy (molecule→senser→procduct→activator→product)
    - Sense environment; produce effect (ex. Smell bread→salivate)
- Design: design specification→simulation modification
    - Apple omnigrapho
    - Microsoft visio
    - Cell modeller
    - Jupyter (anaconda package)
    - Neuvidiaflex
- Create: Make your prototype
    - Gen9 (DNA synthesis company)
    - Twist (DNA synthesis)
    - Cloudlab (do your experiments in a remote lab)

Policy & Practices in DIY Bio and iGEM – David Lloyd

- Core of HP: Society←→ Lab
    - Social sciences based questions
    - Follows a methodology
    - Demonstrates real world application
    - Meaningful impact on your labwork
    - Discover things you didn't know before
- Outreach $\neq$ P&P (outreach is put out; P&P is bring in)
- Read the judge's notebook on website COMMUNICATE! TARGET AUDIENCE!
    - Make it obvious how you fulfill the criteria
    - Make sure you differentiate P&P vs. outreach
- Be careful of survey design!
    - If you are doing a survey, make sure you make it statistically relevant
    - Good example: Gender Study (Paris Bettencourt 2013)

Mathematical Modelling for SynBio – Brian Ingals

- Physical vs. conceptual
- Mathematical modesl can be mechanistic (description) or predictive (make inferences/extrapolation)
- Mass action kinetics
    - Chemical rxn: X→P
    - Rate constant: k=[P]/[X]
    - Etc.
- Programs that can be used
    - COPASI
    - MATLAB
    - XPPAIIT
    - Mathematica
- Separation of time scales; phase plane analysis
    - Processes slow → treated as frozen in time
    - Processes fast → treated as occurring instantaneously

Wiki & Visual Design in iGEM – Patrick Wu

- Design vs. Art (not the same thing)
    - Design: communicate the same thing to everyone (USABILITY is key!)
    - Art: Can have different interpretations to different people
- Content, accessibility, visual hierarchy, grid layout to organize info
- Interpret data for your audience! Figure captions and descriptions
- Summary page for judges is good for check-boxing

May 16, 2016

- Decided on BioBasic as our peptide synthesis company and ordered all of the peptides
- Ordered full length BBI as well as KTI (as a backup peptide) from Sigma Aldrich
- Wait for ~3 weeks for peptides to come!

June 6, 2016

- Dr. Goodarzi had suggested the idea of reducing our peptide; in the original structure of BBI, our truncated section contains 2 cysteines (which means the possibility of cysteine bridges) (Voss, R.-H., Ermler, U., Essen, L.-O., Wenzl, G., Kim, Y.-M. and Flecker, P. (1996), Crystal Structure of the Bifunctional Soybean Bowman-Birk Inhibitor at 0.28-nm Resolution. European Journal of Biochemistry, 242: 122–131.doi:10.1111/j.1432-1033.1996.0122r.x)
- Literature supports that reduction of our peptide would not affect its radioprotective effects (Gueven N, Dittmann K, Mayer C, Rodemann HP. The radioprotective potential of the Bowman-Birk protease inhibitor is independent of its secondary structure. Cancer Letters. 1998;125:77–82.)
- We chose to use DTT as our reducing agent from literature search

June 10, 2016

Clonogenics 0.1 – cell splitting protocol

- take HTC116 cell line with >50% confluency
- wash cell line twice with 1mL of DMSO
- add 1mL 37C trypsin and incubate for 4 minutes
- add 4mL of media (McCoy's with 10%FBS and 0.5%pen-strep) to each of 4 new plates

- add 3mL media to plate from step 3 to quench trypsin, pipette up and down to separate the cells (ideally we have no clumps in the cells but have them individually suspended in solution
- add 1mL of the cells from step 5 to each plate done in step 4, making sure to seed the cells all over the plate
- incubate at 37C with 5% CO_2 to use for next time (over the weekend)

June 13, 2016

Clonogenic 0.1 – solvation of peptide

- dissolved our heptamer peptide (ALSYPAQ) in McCoy's Media, our peptide was not visible to the naked eye after adding McCoy's Media, so it's soluble(?). We then did a serial dilution with the peptide solution to make varying concentrations

concentration	volume of peptide solution	volume of media	total volume
1200μM	dry peptide	10mL	10mL
150μM	1mL from 1200μM	7mL	8mL
100μM	4mL from 150μM	2mL	6mL
50μM	2mL from 100μM	2mL	4mL

We got scolded...

** Remember for next time/actual experiment: only 2-10μL of drug/peptide treatment (negligable amounts) should be added to cell lines to do experiment instead of incorporating the peptide into our media

Clonogenic 0.1 – Cell counting and seeding protocol

- a ~50% confluent cell line of HCT116 cells
- removed media, washed twice with 25°C DPBS
- add 1mL of 37°C trypsin, incubated for 6min in 37C, 5% CO_2 incubator
- quench effects of trypsin by adding 3mL media (McCoy's Media with 10% FBS and Pen-Strep) pipetted up and down to suspend cells
- put 4mL of solution containing cells into autoclaved 15mL falcon tube and centrifuged at 2500rpm for 5min at 25C
- removed all media, leaving only pellet of cell
- resuspended cells with 1mL media (removed 50μL to look at under the microscope to make sure clumping of cells is minimal)
- pipette 10μL of cell mixture and mixed with 10μL of typan blue; pipetted onto cell counting slide (10μL in each of well A and B)
- put the slide in cell counter:
    - Well A: live cells 9.15×10^5 cells/mL (69% live cells)
    - Well B: live cells 7.04×10^5 cells/mL (81% live cells)

Clonogenic 0.1

- The discrepancy between the two wells is quite big, meaning that we did not mix the cell mixture well before counting. Ideally we would want to work with cell cultures that are 85-100% live cells, so we should be careful of cell death (leaving trypsin on for less time, more cogniscent of bubbles etc) in coming experiments.
- But for the sake of practice we decided to seed the cells from the cell counting experiment (average of well A&B: 809500 cells/mL) by plating 6 plates of each of the following cell counts:

cell count/mL	cell solution volume	media volume	total volume
2000	37μL from 809500cell/mL solution	15mL	~15mL
1000	6mL from 2000cell/mL solution	6mL	12mL
500	4mL from 1000cell/mL solution	4mL	8mL

- 1mL of each solution was added to 4mL media on the plates. (6 plates for each cell count)
- the plates were incubated at 37C and 5% CO2 (will check up on them Friday) OUR CANCER BABIES!!!

June 14, 2016

Clonogenic 1.0

- BBI Treatment of HCT116 Cells - 7mer (ALSYPAQ)
    - Time of Application: 10:55 AM
    - Time of Irradiation: 4:10PM

Plates were treated with BBI by Sid (see put peptide in solution entry from Monday 6/13)

Plate #	Cell Density	BBI Concentration	IR Dosage
1-6	500 cells	0	0 Gy
1-3	1000 cells	10 microMolar	5 Gy
4-6	1000 Cells	30 microMolar	5 Gy
1-6	2000 cells	0	5 Gy

June 15, 2016

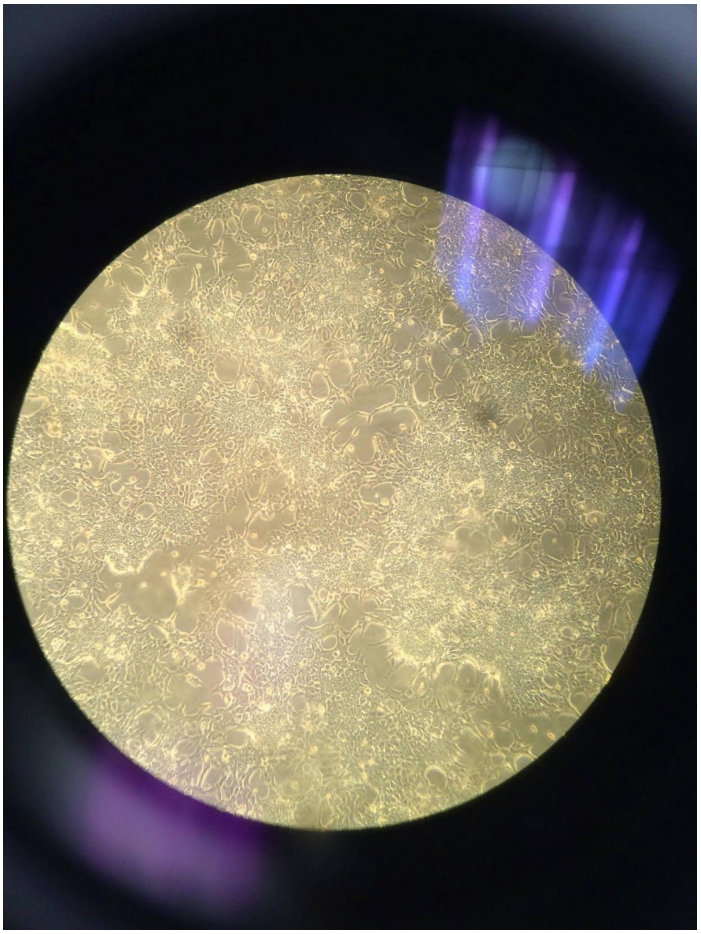
Clonogenic 0.1

- Made 500mL fix solution (3% acetic acid, 8% methanol, 89% water)
- stain solution (0.2% crystal violet, 10% formalin in PBS)
    - could not find formalin to make stain solution, we will get some from Nick Jette, but in the meantime we ordered some

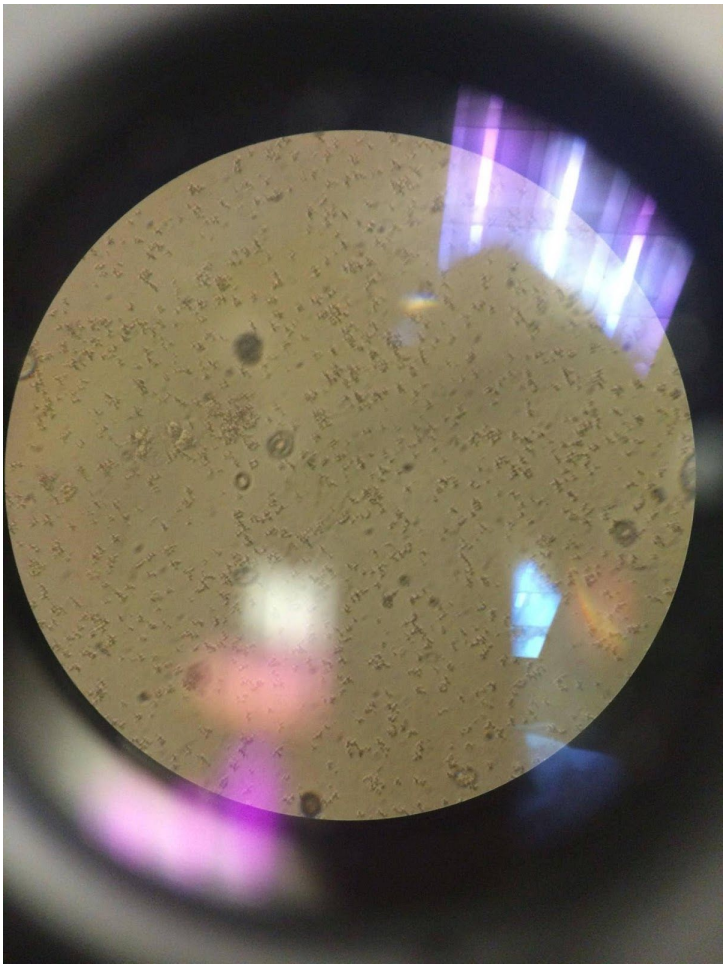
June 16, 2016

Clonogenic 0.1

- Some of our stock cells look very overgrown, so we split some of the less confluent ones, and we are going to throw away the ones that are more overgrown



- Looked at the cells we decided to irradiate and treat. The 1000 plates (treated with BBI and irradiation) looked very strange under the microscope as it had little specks that were not our cells. We suspect it is because our peptides were not soluble in McCoys media and clumped up in solution. So we will try to dissolve the other peptides in DMSO first before applying to our cell cultures. In the meantime, we are trying to salvage our heptamer by centrifuging to see if the peptides will form a pellet so that we can test a few more things on it before we toss it out.



June 17, 2016

Clonogenic 0.1 – Fixing and Staining protocol

- Our staining solution (06/15 entry) was not up to par to the standards as it did not look dark enough. The suspicion is that our crystal violet was not a the right concentration to begin with (as our crystal violet came in solution form instead of powder form), so we will go back and search up the initial concentration of our crystal violet. In the mean time, this practice will be done with crystal violet provided to us by the Susan-Lees Millers lab.
- Media was removed from plates
- 1mL fix solution was added (see entry on June 15); let sit for 2 min, then removed
- 1mL stain solution was added (see entry on June 15); let sit for 5 min, then removed
- deionized water was added to each plate (covers surface of the plate) to rinse the cells, removed

Clonogenic 0.1

- looked at stained colonies
    - the 500 plates had more colonies than the other 2 types of plates
    - 1000 plates had very little to no colonies
    - 2000 plates had about the same amount of colonies than 500 plates, but the colony sizes are much smaller

June 20, 2016

Clonogenic 0.2

- before we start to do our official trials, we needed to do another practice to familiarize ourselves with techniques
    - Learned that the P1000 pipettes were very effective in breaking up cells
- counted and seeded 48 plates with varying cell counts (x6 plates for each manipulation)

Drug (DMSO) Treatment Volume	0Gy Radiation	5Gy Radiation
0µL	sd. 500	sd. 2000
3µL	sd. 500	sd. 1000
6µL	sd. 500	sd. 1000
9µL	sd. 500	sd. 1000

June 21, 2016

Clonogenic 0.2

- Treated cells with varying volumes (Table15) of filter-sterilized DMSO as a carrier control as well as irradiated all our plates.
- Now to just wait for our babies to grow in the incubator!

Clonogenic 0.2 - For Thursday (10AM)

- More cell count and seeding practice
- counting practice plates from last friday in the cell colony counter and doing statistics on them
- setting up the vacuum media aspirator (maybe)

June 23, 2016

Clonogenic 0.1 - Colony Counting Protocol

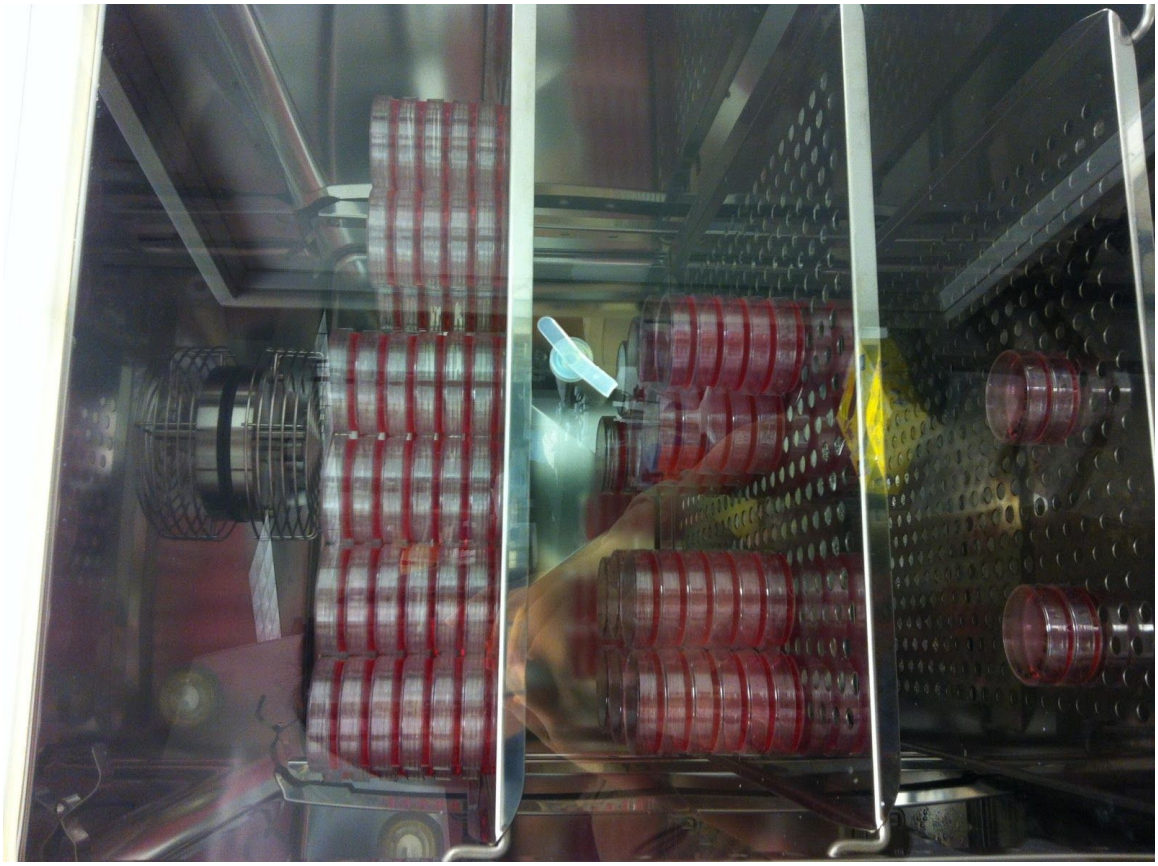
- Open the computer, select Colcount and the password is dnapk*atm
- Once on the desktop screen select colcount from the desktop
- Once the application opens click start, select 50mm UpperPosition (7E2) and then click Load
- Select Cell present and hit browse - use either Nick(good) or small colonies
- Insert the blank with no tip and it will start warming up.
- Optional - Save at desktop and make new folder every day
- Grab the plates with colony and start counting
- **Happy Counting**

Plates	500	1000	2000
1	46	9	22
2	84	1	10
3	63	12	22
4	53	13	8
5	42	7	19
6	43	7	8

June 27, 2016

Clonogenic 0.2

- Cells were trypsinized and counted using the Bio-Rad counter and plates.
    - Well A: 1.17 x 10^7 cells/ml (76% alive)
- 36 x 1000 cell plates and 36 x 500 cell plates were seeded with their respective amount of cells. 1ml of cell culture was added to 4 ml of McCoy's media with FBS and Pen-Strep.



June 29, 2016

HCT116 Clonogenics 1.0

- Our phosphorylated BBI peptide as well as our control scramble peptide came in!
- We tried to seed our 168 plates today (see google docs clonogenics trial 1), but in our cell count, it turns out only 12-14% of our cells are alive??! (even though we redid it 3 times)
    - we suspect that the cell counter was acting up as non of the other people's samples were working either. But either way those results are unusable, so we will have to push this off till next Monday to seed as this Friday will be Canada Day.

July 4, 2016

HCT116 Clonogenics 1.0

- Used stock cell plate with cells split on June 29th, also tried counting with cells from 2 weeks ago with 1000 seed but decided not to use them because cell survival was 9%
- Cell survival percentage of the stock solution used is average of 53%, decided to go ahead with the trials as we suspect it is our cell counting protocol that needed tweaking as opposed to our cells actually being fifty percent dead
    - Seeded 100 plates with 500 cells/plate
    - seeded 100 plates with 1000 cells/plate

July 5, 2016

HCT116 Clonogenics 1.0

- Plan of our clonogenics trial

	plates #	Volume added (uL)	Radiation(Gy)	Seeding	# Plates	Time drugged	Time irradiated
Control (DMSO+DTT)	1	0	0	500	3	11:20	
	2	3	0	500	3	11:21	
	3	6	0	500	3	11:25	
	4	9	0	500	3	11:28	
	5	0	5	1000	3	11:20	5:52
	6	3	5	1000	3	11:21	5:32
	7	6	5	1000	3	11:25	5:42
	8	9	5	1000	3	11:28	5:42
9mer (S)	1	0	0	500	3	11:30	
	2	3	0	500	3	11:32	
	3	6	0	500	3	11:35	
	4	9	0	500	3	11:40	
	5	0	5	1000	3	11:30	6:21
	6	3	5	1000	3	11:32	6:02
	7	6	5	1000	3	11:35	6:25
	8	9	5	1000	3	11:40	6:05
KSCl BBI	1	0	0	500	3	11:45	
	2	3	0	500	3	11:46	
	3	6	0	500	3	11:48	
	4	9	0	500	3	11:50	
	5	0	5	1000	3	11:45	5:52
	6	3	5	1000	3	11:46	5:36
	7	6	5	1000	3	11:48	6:02
	8	9	5	1000	3	11:50	5:36
Big BBI	1	0	0	500	3	12:00	
	2	9	0	500	3	12:02	
	3	18	0	500	3	12:05	
	4	27	0	500	3	12:08	
	5	0	5	1000	3	12:00	5:25
	6	9	5	1000	3	12:02	5:19
	7	18	5	1000	3	12:05	5:25
	8	27	5	1000	3	12:08	5:19
KTI	1	0	0	500	3		
	2	12	0	500	3	11:09	
	3	24	0	500	3	11:13	
	4	36	0	500	3	11:14	
	5	0	5	1000	3		6:21
	6	12	5	1000	3	11:23	6:17
	7	24	5	1000	3	11:21	5:05
	8	36	5	1000	3	11:20	6:13
Scramble	1	0	0	500	3		
	2	3	0	500	3	11:29	

	3	6	0	500	3	11:32	
	4	9	0	500	3	11:33	
	5	0	5	1000	3		5:56
	6	3	5	1000	3	11:37	6:20
	7	6	5	1000	3	11:40	6:27
	8	9	5	1000	3	11:41	5:47
P-Tyrosine 9mer (S)	1	0	0	500	3		
	2	3	0	500	3	11:52	
	3	6	0	500	3	11:50	
	4	9	0	500	3	11:48	
	5	0	5	1000	3		5:56
	6	3	5	1000	3	11:55	6:13
	7	6	5	1000	3	11:57	6:10
	8	9	5	1000	3	11:59	5:47

July 11, 2016

H2AX Protocol

- The cells are taken from 80-100% confluent flask and then are plated into desired number of glass slides.
- The plates are incubated in order to reach 100% confluency.
- Once the cells have reached 100% confluency they are divided into different treatment groups.
- The cells are treated with their respective treatments and following treatment they are washed with PBS at a certain period of time. (Note: The washes are poured onto the side, not directly on top of the slide.)
- Once the cells are washed, 100uL of fixative solution (3% w/v Paraformaldehyde, 2% w/v sucrose, 1X PBS). The cells are fixed for 10 min at room temperature.
- The cells are then washed with PBS twice after the fixing them.
- The cells are then stored for next day for antibody staining.
- Fixed, washed cells are then permeabilized by adding 100uL of Triton X-100 on the coverslip.
- Cells are then washed twice with PBS.
- Fixed and Permeabilized cells are then incubated with 100uL of primary antibody (H2AX, 53BP1 and H3S10p) diluted in PBS + 2% (w/v) BSA for 1 hour at room temperature.
- Cells are then washed three times with PBS and then incubated with 100uL of secondary antibody (Alexa-488, Cy3, DAPI) diluted in PBS + 2% (w/v) BSA for 20 min at room temperature in dark.
- Cells are then incubated with ~2mL of 4',6-diamidino-2-phenylindole (DAPI) diluted in PBS for 5-10 min at room temperature.
- Coverslips are then taken out and mounted onto the slides using Vectashield mounting media. The coverslip is then sealed with nail polish to keep it fixed at its position.
- Once the slides are ready, they are taken to the Axiovert microscope to count the number of foci.

July 12, 2016

HCT116 Clonogenics 1.0

- the cultures with p(y) 9mer looks more yellow than other plates, perhaps indicating more colonies (more to come in 2 weeks)

July 15, 2016

HCT116 Clonogenics 1.0

- We had set up a vacuum to suck off the media as well as treat our plates with fix and stain solution. Colonies were counted using ColCount colony counter.

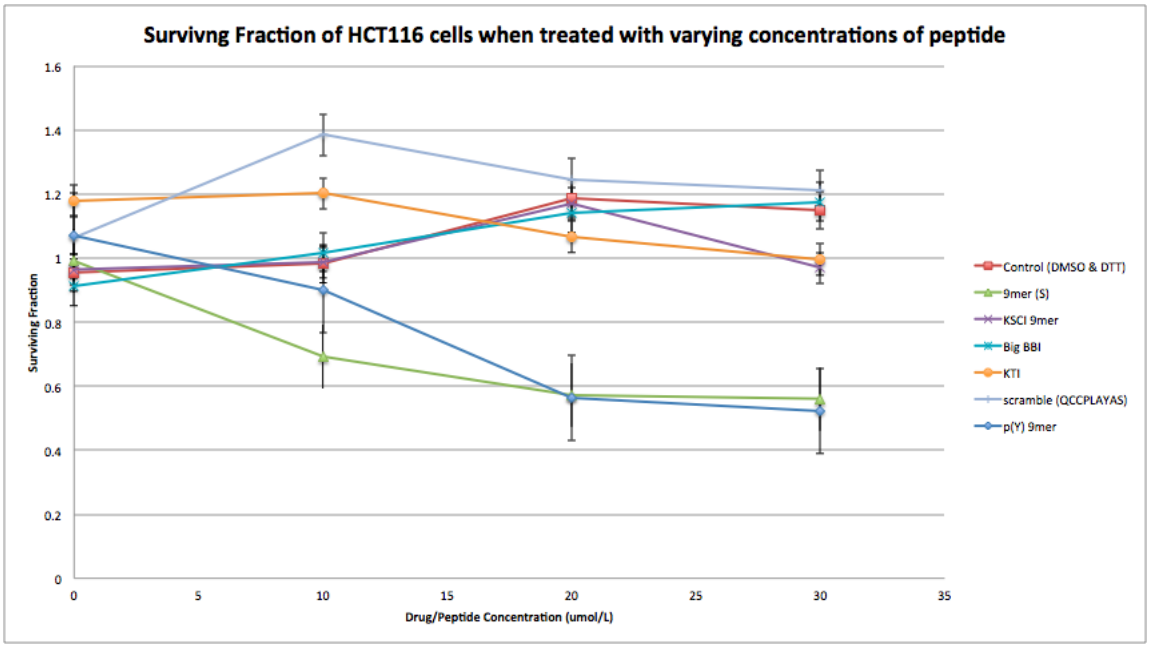
	Concentration (µmol/L)	Radiation(Gy)	Seeding	Colony Count			Treatment Concentration	Surviving Fraction	Trial PE
Control (DMSO+DTT)	0	0	500	250	200	168	0	0.955085482	**0.431375**
	10	0	500	178	242	215	10	0.98135806	
	20	0	500	237	311	220	20	1.186902347	
	30	0	500	223	270	250	30	1.148266203	
	0	5	1000	16	24	15	0	0.042499759	
	10	5	1000	37*	18		10	0.041727036	
	20	5	1000	31*	8	16	20	0.027818024	
	30	5	1000	20	28	6	30	0.041727036	
9mer (S)	0	0	500	208	220		0	0.992176181	
	10	0	500	77	191	180	10	0.692359703	
	20	0	500	122	129	120	20	0.573360379	
	30	0	500	106	117	140	30	0.560996813	
	0	5	1000	20	12	9	0	0.031681638	
	10	5	1000	17	30	27	10	0.057181493	
	20	5	1000	22	7	3	20	0.024727132	
	30	5	1000	13	9	26	30	0.037090698	
KSCl BBI	0	0	500	194	221	209	0	0.964358157	
	10	0	500	212	209	218	10	0.987539844	
	20	0	500	247	268	242	20	1.169902444	
	30	0	500	227	201	199	30	0.968994494	
	0	5	1000	48*	66*	50*	0		
	10	5	1000	23	6	13	10	0.032454361	
	20	5	1000	5	17		20	0.025499855	
	30	5	1000	85*	11		30	0.025499855	
Big BBI	0	0	500	184	202	204	0	0.911813001	
	10	0	500	250	198	211	10	1.018448759	
	20	0	500	256	236		20	1.140538974	
	30	0	500	225	290	246	30	1.176084227	
	0	5	1000	19	48*	11	0	0.03477253	
	10	5	1000	22	15	28	10	0.050226987	
	20	5	1000	18	10	23	20	0.039408867	
	30	5	1000	50	21	28	30	0.076499565	
KTI	0	0	500	247	267	250	0	1.180720564	
	10	0	500	251	286	241	10	1.202356805	
	20	0	500	222	236	232	20	1.066357578	
	30	0	500	198	240	206	30	0.995267072	

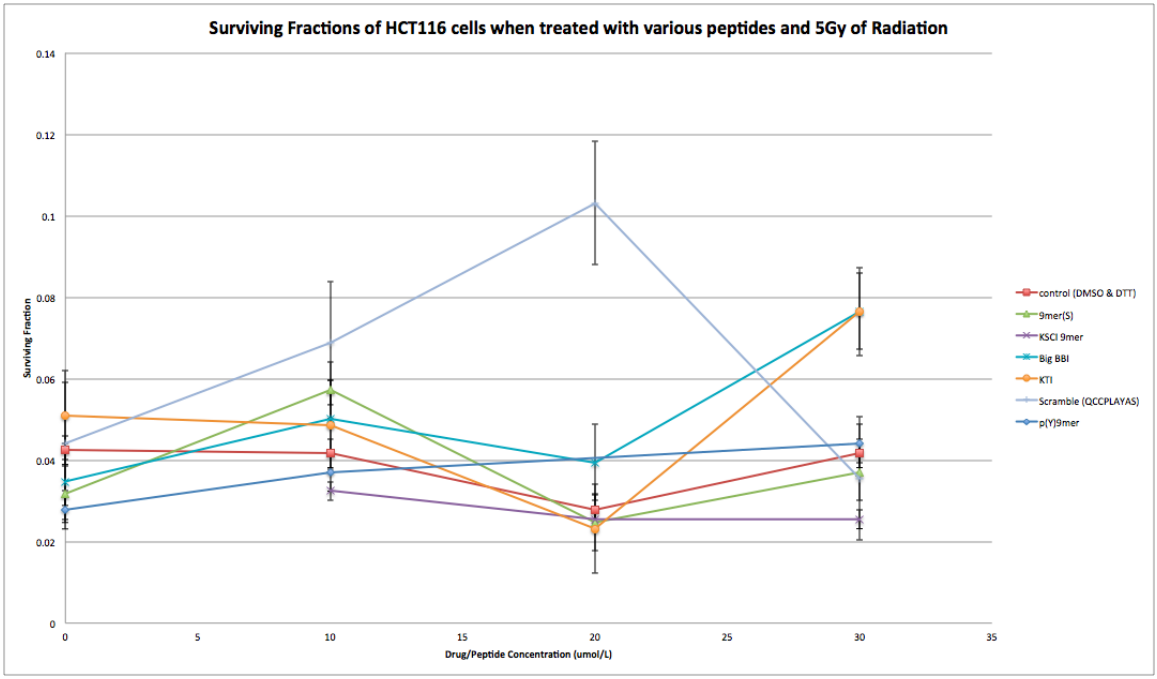
	0	5	1000	22	43*		0	0.05099971	
	10	5	1000	21	79*		10	0.048681542	
	20	5	1000	30	83*	33*	20	0.023181686	
	30	5	1000	27	30	42	30	0.076499565	
Scramble	0	0	500	218	227	243	0	1.063266686	
	10	0	500	278	283	335	10	1.384719405	
	20	0	500	271	284	252	20	1.247174732	
	30	0	500	248	295	240	30	1.210084034	
	0	5	1000	17	31	9	0	0.044045204	
	10	5	1000	25	33	31	10	0.068772337	
	20	5	1000	36	53		20	0.103158505	
	30	5	1000	21	13	12	30	0.035545253	
P-Tyrosine 9mer (S)	0	0	500	241	216	235	0	1.069448469	
	10	0	500	180	226	176	10	0.899449435	
	20	0	500	107	126	131	20	0.562542258	
	30	0	500	107	108	123	30	0.522360668	
	0	5	1000	10	14		0	0.027818024	
	10	5	1000	13	19		10	0.037090698	
	20	5	1000	51*	51*		20		
	30	5	1000	37	8	12	30	0.044045204	

>blank spaces indicates the plates were contaminated in the process of obtaining the data, as such data was not obtained and these plates were excluded from counting.

HCT116 Clonogenic 1.0 - Graphs

- Graph 1 is of plates seeded with just 500 cells and treated with just variuos peptides and no radiation to see if peptides inherently have toxicity to our cells. Data was created in excel with different treatments overlayed on top of one another. Error bars represent excel calculated standard error for those particular data points.
- Graph 2 is of plates seeded with 1000 cells and treated with various peptides as well as 5Gy radiation to see if our peptides confer radioprotection to our cells. Data was created in excel with different treatments overlayed on top of one another. Error bars represent excel calculated standard error for those particular data points. (note the scale difference between the two graphs)





July 18, 2016

<u>HCT116 Clonogenics 1.0 - Discussion</u>

- None of the peptides we intended to test showed significant radioprotection compared to out control with just DMSO and DTT (the solvent we dissolved our peptide in)
- BBI and KTI were interesting in that at 30µmol/L they seem to be conferring radioprotection to our cell-line compared to the control; but this relationship does not seem linear (no apparent linear correlation between application of the peptide and surviving fraction). Also cannot be sure of the significance of this observation due to the small increase that is apparent (being concious of the scale on the second graph)

- our scramble peptide seemed to increase the surviving fraction of irradiated cells <20μmol/L, but at 30μmol/L seemed to do nothing to help the surviving fraction of our cell-line. Only 2 datapoints were used in the calculation of the surviving fraction of cells treated with 20μmol/L scramble peptide so the error on this observation could be large--> requires more in-depth statistical analysis before conclusions.
- The Serine 9mer as well as the p(Y)9mer seemed to be killing our cells/making them unable to reproduce, suggesting toxicity against HCT116 cells or cancer cells in general; while the rest of our peptides were comparable to the control treatement in terms of toxicity (or lack thereof)
- KTI protein as well as our scramble peptide showed interesting results on cells that did not undergo irradiation, in that those peptides seem to increase the surviving fraction on our cell-line

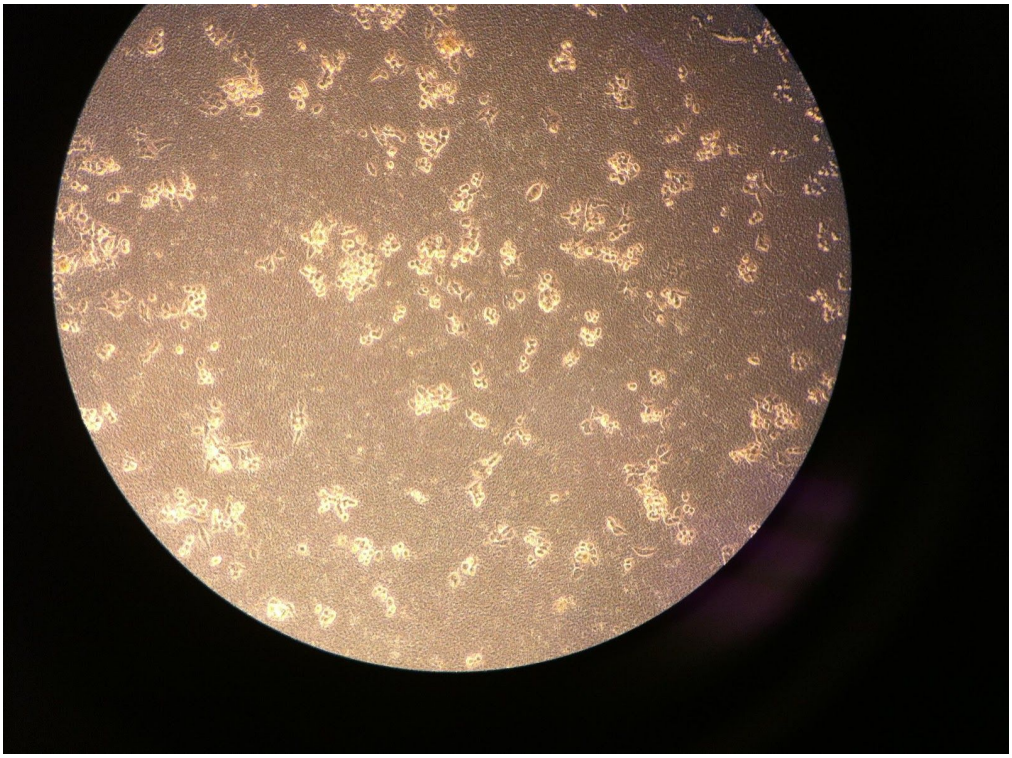
Next steps: Trypan Blue Assay Protocol

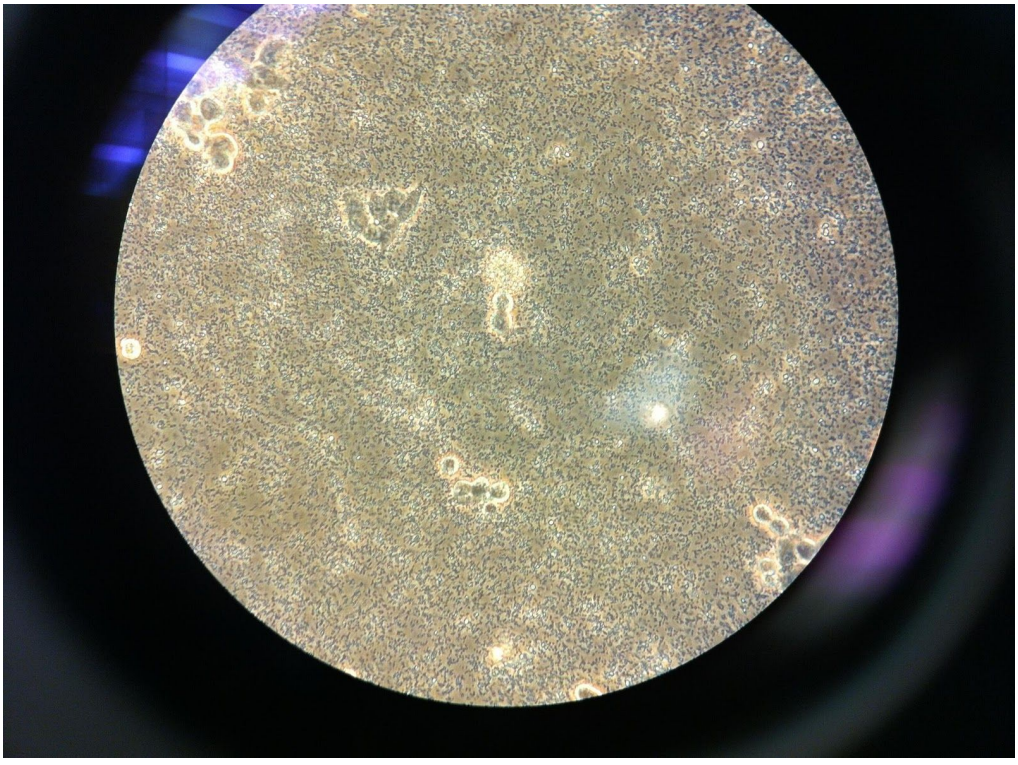
- Discussed next steps with our mentors and decided to do a Trypan Blue survivability assay in the interest of time. Trypan Blue Assay should be able to give us some analyzable results in the time-frame of a week
    - Grow, treat and irradiate the cell line as needed to test timing as well as different peptides (splitting cells without being concious of the number seeding)
    - Aspirate the media and place it in an autoclaved 15mL falcon tube
    - wash cells iwth DPBS and Trypsinize to lift cells off the plate, quench, then pipette the solution into the same 15mL falcon tube
    - Mix well and stain with trypan blue stain.
    - Quickly count total cells and live cell fraction with BioRad 500 cell counter. Record percentage of live cells and tabulate for analysis of survivability.

July 20, 2016

HCT116 Trypan Blue 1.1

- attempeted to count cells for trypan blue assay, but after trypsinizing for longer than usual (8min), the cells at the botom of the plate was unwilling to lift off the plate when viewed under a microscope. Seemed like there was a carpet of other smaller material around the cells that is trapping the cells to the bottom of the 60mm plate. Media was normal and was not cloudy (which is why we were unsure about what to think.
- Consulted this observation with our advisor, who announced that it was likely contamination. As a result all plates were discarded (all plates seemed to see the same situation) and the incubator was cleaned out as the water in the incubator was getting gross (which might have been the source of the contamination to begin with)
- Our HCT116 stock cells do not seem to be affected





July 26, 2016

<u>H2AX Training 0.1</u>

- Learned how to fix the cells

- Learned how to do antibody staining

July 27, 2016

H2AX Training 0.1

- Learned how to prepare slides with mounting media and nail polish

July 28-29, 2016

H2AX Training 0.1

- Learned how to set-up the microscope and use different filters

August 2, 2016

1BR3 Clonogenic 1.0 – Splitting protocol

- New Materials
    - Media was composed of MEM (no extraneous nutrients), 15% FBS, 1% Penstrep, 2mM L-Glutamine (Stock is 200mM or 100x)
    - used 0.25% trypsin
    - used DPBS with no additives

1. Fibroblasts were looked at under the microscope to ensure over 70% confluency
2. Aseptic technique practiced to a tee (turn on hood 15 min before using, warm up media and trypsin in the 37c water bath, spray and wipe down everything with ethanol, ensure we have sterile/autoclaved 15mL falcon tubes)
3. wash 2 times with DPBS (making sure to pipette PBS on the side of the flask away from our cells)
4. add 3ml trypsin in each flask, incubate at 37c for ~5min
5. bump side of flask to ensure all cells are rounded and deadhered (check under the microscope)
6. add media to quench trypsin effects (around 6mL)
7. pipette media around the flask to "wash" down the surface (making sure declumped and deadhered)
8. remove all media and place in a 15mL falcon tube
9. centrifuge tubes at 2500rpm, 4c, for 3 minutes; make sure there is a pellet (should be visible for a T75 flask)
10. while the tubes are being centrifuged, fill new flasks with 14-18mL of media (T75 flasks)
11. remove the falcon tubes from centrifuge, aspirate the media
12. resuspend cells in 6mL media (for a 1:3 split) or 8mL media (1:4 split)
13. add 2mL into each new flask
14. tilt back and forth for an even spread
15. place back into incubator and do not touch the cells again (to not disturb) until you need to use them next

H2AX Training 0.1

- Learned how to count foci
- Advised to use blind counting technique for actual experiment

August 5, 2016

1BR3 Clonogenic 1.1

- Trypsinized/ Lifted a T75 flask of 1BR3 feeder fibroblasts(passage #: 12; 80% confluency) and added to 10mL of media
- Irradiated cells at 35 Gy of radiation (^{134}Cs source, 85 Gy/min)
- Diluted cells to make 110mL of cell solution

- Seeded a layer of feeder cells on 108x60mm plates by pipetting 3mL of media and 1mL of seeder cells
- Placed in 37 ◦ C, 5% CO2 overnight

August 6, 2016

1BR3 Clonogenic 1.1

- Counted 1BR3 cells from our T25 flask stock
    - Well A: $5.98x10^5$ (52% live); Well B: $6.41x10^5$(48% live)
- Dilution and seeding of live 1BR3 cells to feeder plates

August 7, 2016

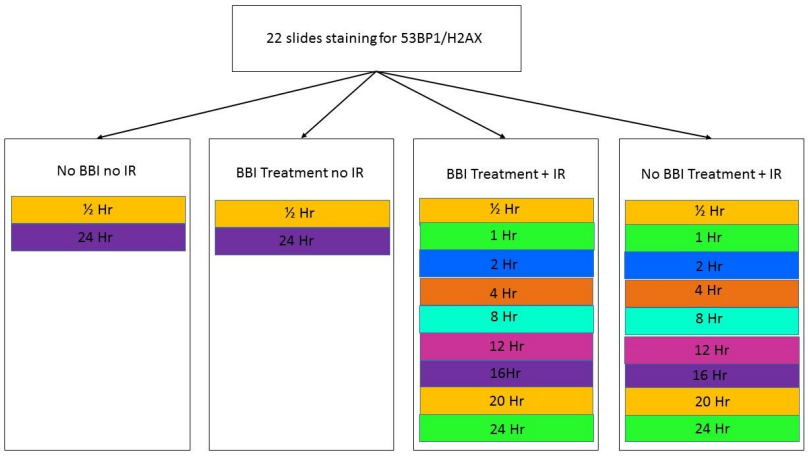
1BR3 Clonogenic 1.1

- Treatment with various peptides (big BBI, 9mer, p(Y) 9mer, KTI, DTT+DMSO, no treatment control)
    - Peptides were added to a final concentration of 20uM
- Irradiation with 3 Gy of radiation according to original plan and seeding
- Placed in Placed in 37 ◦ C, 5% CO2

August 8, 2016

H2AX Assay 1.1

- Cells were plated on glass slides (18) and after they reached 100% confluency they were put into different treatments



- The procedure was followed with 2Gy of radiation treatment
- Number of foci were counted twice using bling counting technique each time.

Treatment	Time	Count #1	Count #2	Average
BBI+, IR-	0.5	0.77	0.7	0.735
BBI-, IR-	0.5	1	0.82	0.91
BBI+, IR+	24	9.13	8.97	9.05

BBI-, IR-	2	21.13	20.7	20.915
BBI-, IR+	8	8.17	8.83	8.5
BBI-, IR-	24	6.06	6.13	6.1
BBI+, IR-	24	0.88	1.36	1.12
BBI+, IR+	2	19.59	16.7	18.145
BBI-, IR+	24	3.82	2.59	3.205
BBI+, IR+	8	7.41	6.24	6.825
BBI-, IR+	4	11.18	12.3	11.74
BBI-, IR+	0.5	29.5	32.03	30.77
BBI-, IR+	16	3.6	2.77	3.19
BBI+, IR+	16	1.15	1.37	1.26
BBI+, IR+	0.5	29.6	26.7	28.15
BBI+, IR+	4	12.7	8.1	10.4
BBI+, IR+	1	29.73	28.4	29.07
BBI-, IR+	1	32.1	30.8	31.45

August 11, 2016

1BR3 Clonogenic 1.1 – Contamination

- Found our stock flasks are contaminated
- Cleaned out our incubator and streaked reagents (media, DPBS, contaminated plate) on some LB plates to see what is causing the contamination
- Obtained more cells from Dr. Goodarzi – will be able to receive them in the near future

August 12, 2016

1BR3 Colonogenic 1.1 – Contamination results

- Non of our reagents were found to be contaminated through streaking on LB
- Thought about the possibility of our trypsin being contaminated but eliminated the possibility as trypsin is a protease and cell should not grow there
- Contamination most likely due to incubator not being clean, so we thermal cycled our original incubator, and in the meantime we have switched to using a different incubator to avoid further contamination

August 15, 2016

Big Team meeting

- Presented biotarget results to the whole team and all of our advisors

- Dr. Goodarzi, after seeing our presentation, gave us some suggestions on what to do going forward
    - Suggested using lower Gy (ex. 0.25, 0.5, 1.0) to test for hyper-radiosensitivity that is observed at low Gy → more applicable to project as typically astronauts receive radiation in 0.25 Gy range (Short S.C., Woodcock M., Marples B., 2003. Joiner M.C. Effects of cell cycle phase on low-dose hyper-radiosensitivity International Journal of Radiation Biology, 79 (2) , pp. 99-105.)
    - Suggested altering the concentration of peptide added to our H2AX assay to see the effects of altering concentration on DSB repair
    - Suggested looking at more timepoints after radiation for H2AX assay to get a better trend of looking at when BBI works on our cells
    - Will be providing more 1BR3 cells for us to work with; Interested in looking at a comparison of how our peptides work in cancer cells vs. primary fibroblasts

August 18,2016

1BR3 Clonogenic 1.2

- Trypsinized/ Lifted a T125 flask of 1BR3 feeder fibroblasts(passage #: 12; 80% confluency) and added to 10mL of media
- Irradiated cells at 35 Gy of radiation (^{134}Cs source, 85 Gy/min)
- Diluted cells to make 225mL of cell solution
- Seeded a layer of feeder cells on 220x60mm plates by pipetting 3mL of media and 1mL of seeder cells
- Placed in 37 ◦ C, 5% CO2 overnight

August 19, 2016

- Found a patent on a product that is very similar to ours with the inventors Minnie McMillan and Lynn E. Foster (Patent: http://www.google.ch/patents/US20150050250)

1BR3 Clonogenic 1.2

- Counted 1BR3 cells from our T25 flask stock
    - Well A: 4.02x10^5 (100% live); Well B: 2.92x10^5(100% live)
- Dilution and seeding of live 1BR3 cells to feeder plates
    - To make 115 mL cell concentration of 700 cells/mL for our 4 Gy radiation plates

$$\bar{x} = \frac{4.02\times10^5+2.92\times10^5}{2} = 3.47\times10^5$$

$$(347000\tfrac{cells}{mL})(x\ mL) = (700\tfrac{cells}{mL})(115\ mL)$$
$$x = 232\mu L$$

Radiation (Gy)	Seeding concentration (Cells/mL)	Volume for Serial Dilution		
		Vol from previous concentration (mL)	Vol Media (mL)	Total Volume (mL)
4	700			115
2	500	75	30	105
1	250	65	60	125
0.5	200	85	21	106
0.25	175	66	9	75

0	150	35	5	40

- Plates previously seeded with feeder layer (August 18) were plated 1mL of cells diluted to the specified concentrations in the table above according to intended radiation treatment
- Plates treated with various types of peptides (big BBI, 9mer, p(Y) 9mer, KTI, DTT+DMSO, control) (4-5PM)

August 20, 2016

1BR3 Clonogenic 1.2

- Cells irradiated with 137Cs source (8.5Gy/min) and designated Gy (0.25/1.7s, 0.5/3.5s, 1/7s, 2/14.1s, 4/28s) at 8AM (total incubation with peptides before radiation: 17hr)

August 22, 2016

1BR3 Clonogenic 1.2 - Contamination

- Plates for both the Clonogenic assay and the H2AX assay were found to be contaminated when looked at under the microscope (40x magnification); both assays are not able to continue
- Contaminated plates were disposed of; kept a few plates for reference to test the source of contamination

August 23, 2016

1BR3 Clonogenic 1.2 - Contamination

- Informed Nick Jette regarding contamination, he suggested we stop working out of the refrigerator as our chassis team, which may be the root of our contamination
    - He will contact Dr. Goodarzi to see if we will be able to use some of his lab space so that we do not get any further contamination
- Look for the root of the contamination by plating various reagents on 60mm plates
    - New unopened MEM (control plate)
    - Old media (5mL)
    - DPBS (1mL) + MEM (4mL)
    - Trypsin (1mL) + MEM (4mL)
    - MEM (5mL) + assortment of mBBI peptides
    - MEM pipetted by Nick (to test technique)
    - MEM pipetted by Nilesh (to test technique)
    - MEM pipetted by Sid (to test technique)
    - MEM left open in the incubator (to test bacterial growth in incubator
- Plates were left in the incubator (37C, 5% CO2) overnight

August 24, 2016

1BR3 Clonogenic 1.2 - Contamination Results

    - No growth observed in plates containing new or old media, DPBS, media plated by all experimenters
    - Bacterial growth found in plates containing trypsin (viewed under 100x)
- More cells (1 x T75 flask (50% confluent as of today)) will be obtained (we will most likely when our trypsin comes in)
- Wendy (lab technician) donated 5 x T75 flasks as well as 60 x T25 flasks to us

August 25, 2016

Oxidized vs Reduced BBI - Immunofluorescence (h2ax)

- 4 plates of 1BR3 cells were allowed to reach 100% confluency.

- Oxidized BBI at 30uM of concentration were administered to two plates, while the other two received reduced BBI.
- The BBI was reduced with DTT, and it was ensured that a concentration of 500uM or higher of DTT was maintained in the plate.
- Cells were then radiated at 2Gy, and fixed at 4hrs and 8hrs using PFA.

August 25, 2016

Oxidized vs Reduced BBI - Immunofluorescence (h2ax)

- Immunofluorescence protocol was followed for antibody staining.
- The coverslips were then mounted on slides and the number of foci were counted using axiovert microscope.

Time/BBI	Oxidized BBI	Reduced BBI
4hrs	10.96	10.3
8hrs	5.43	5.86

September 1, 2016

Dosage Study

- 20 Plates of 1BR3 cells were allowed to reach 100% confluency
- Non-reduced BBI at 30uM concentration was administered to cells 6 hrs prior to radiation.
- All the cells/plates were fixed at 15 min using PFA in order to examine the quenching abilities of BBI.

September 2, 2016

1BR3 Clonogenic 2.0

- Trypsinized/ Lifted a T75 flask of 1BR3 feeder fibroblasts(passage #: 12; 80% confluency) and added to 10mL of media
- Irradiated cells at 35 Gy of radiation (^{134}Cs source, 85 Gy/min)
- Diluted cells to make 110mL of cell solution
- Seeded a layer of feeder cells on 108x60mm plates by pipetting 3mL of media and 1mL of seeder cells
- Placed in 37 ◦ C, 5% CO2 overnight

Dosage Study

- Cells were then stained using a primary antibody 53BP1 (The shame)
- The coverslips were then mounted on the slides and the number of foci were counted using axiovert microscope.

BBI/Gy	0	0.5	1	2
0	0.72	3.63	17.2	13.33
10	24.2	6.875	21.43	16.43
30	1.57	15.97	28.73	25.5

60	18.83	13.03	16.47	0.677
90	2.3	1.472	26.76	16.19

September 3, 2016

1BR3 Clonogenic 2.0

- Counted 1BR3 cells from our T25 flask stock
  - Well A: 2.56×10^5 (93% live); Well B: 2.01×10^5(98% live)
- Dilution and seeding of live 1BR3 cells to feeder plates
  - To make 115 mL cell concentration of 700 cells/mL for our 4 Gy radiation plates

$$\bar{x} = \frac{2.56\times10^5+2.01\times10^5}{2} = 2.28\times10^5$$

$$(228000\tfrac{cells}{mL})(x\ mL) = (700\tfrac{cells}{mL})(57\ mL)$$
$$x = 175\mu L$$

Radiation (Gy)	Seeding concentration (Cells/mL)	Volume for Serial Dilution		
		Vol from previous concentration (mL)	Vol Media (mL)	Total Volume (mL)
4	700			57
2	500	37	15	52
1	250	32	31	63
0.5	200	43	10	53
0.25	175	33	4	37
0	150	17	3	20

- Plates previously seeded with feeder layer (September 2) were plated 1mL of cells diluted to the specified concentrations in the table above according to intended radiation treatment (4-5 PM)

September 5, 2016

1BR3 Clonogenic 2.0

- Treatment with various peptides (big BBI, 9mer, p(Y) 9mer, KTI, DTT+DMSO, no treatment control)
  - Peptides were added to a final concentration of 20uM
  - Done at 7 AM
- Irradiation with various Gy of radiation according to original plan and seeding
  - 1 PM (for total 6 hr. incubation with peptide)
- Placed in Placed in 37 ◦ C, 5% CO2

September 16, 2016

1BR3 Clonogenic 2.0

- Contamination found in all of clonogenic plates again. Will not have time to do another clonogenic trial
- Suspect the incubator being the problem as it was found that the air filter has not been changed on the incubator for a while

- setting up a collaboration with Goodarzi lab to use their incubator for future H2AX (will not have time to do clonogenics and deemed that if results are found of H2AX, it should be pretty conclusive)

September 17, 2016

Immunofluorescence Assay 1.2

- 22 plates of cell were allowed to reach 100% confluency
- The plates were administered non-reduced BBI at 30uM concentration 6hrs prior to radiation.
- Cells were irradiated and the fixing was done using PFA at regular intervals - 0.5, 1,2,4,8,12,16,20,24hrs. (Two more time points are added to the previous assay).
- Once the cells were fixed, antibody staining was done using 53BP1 primary antibody and secondary antibody (FITC and Cy3) were used for visualization.

September 18, 2016

Immunofluorescence Assay 1.2

- Once the cells were fixed, antibody staining was done using 53BP1 primary antibody and secondary antibody (FITC and Cy3) were used for visualization.
- The coverslips were then mounted on the slides.

September 29, 2016

Immunofluorescence Assay 1.2

- The number of foci were counted using axiovert microscope.

A	B	C	D	E	F
0	Time (Hr)	BBI-/IR-	BBI+/IR-	BBI+/IR+	BBI-/IR+
1	0.5	0.58	0.37	38.4	40.1
2	1			34.7	39.65
3	2			28.85	32.55
4	4			25.27	27.95
5	8			14.57	17.48

6	12			5.57	8.22
7	16			4.9	5.85
8	20			1.74	2.65
9	24	0.8	0.72	1.66	2.43

Mouse Trials

TPost- Transdermal Patch Application Observations:

Day 2: September 9, 2016:

- **Control Treatment:**
  - Control 1: The patch was detached. There were no behavioral changes that were observed that indicated distress, pain, etc.
  - Control 2: The patch was still adhered to the skin. There were no behavioral changes that were observed that indicated distress, pain, etc.
  - Control 3: The patch was detached. There were no behavioral changes that were observed that indicated distress, pain, etc.
- **Chassis Treatment (*Bacillus subtilis*):**
  - *B. subtilis* 1 and 2: The patch was detached. A small wound was observed on the left top corner where the patch was ripped off.
  - *B. subtilis* 3: The patch was still adhered to the skin. There were no behavioral changes that were observed that indicated distress, pain, etc.
  - *B. subtilis* 6: The mouse died in first 24h of the study.
  - BBI 1: The patch was detached. There were no behavioral changes that were observed that indicated distress, pain, etc.
  - BBI 2: The patch was still on. There were no behavioral changes that were observed that indicated distress, pain, etc.
  - BBI 3: The patch was still on. There were no behavioral changes that were observed that indicated distress, pain, etc.
  - BBI 6: Died in first 24h of the study

Day 5: September 12, 2016:

- **Control Treatment:**
  - Control 4: The patch was detached. There were no behavioral changes that were observed that indicated distress, pain, etc.
  - Control 5: The patch was detached. There were no behavioral changes that were observed that indicated distress, pain, etc.

    - Control 6: The patch was detached. There were no behavioral changes that were observed that indicated distress, pain, etc.
- **Chassis Treatment (*Bacillus subtilis*):**
    - *B. subtilis* 4: The patch was detached. The mouse died immediately before blood draw
    - *B. subtilis* 5: The Patch was still on. There were no behavioral changes that were observed that indicated distress, pain, etc.
- **BBI Treatment:**
    - BBI 4: The patch was detached. Some movement was observed after administration of ketamine. So, isoflurane was administered through inhalation before blood draw.
    - BBI 5: The patch was detached. There were no behavioral changes that were observed that indicated distress, pain, etc.

Immunohistochemical Staining:

Experimental Details and Rationale:
This technique was employed to study any possible immunological responses induced by the transdermal patch, the bacteria *Bacillus subtilis*, and mBBI. Mice skin tissue samples were obtained from 9 mice at day 2 (samples 1-3 for all treatments) and the rest of the mice samples were obtained on day 5 (samples 4-6 for all treatments) of the study. The skin tissue samples were then fixed, sectioned, and stained. Due to lack of training and materials, members of the Jenne lab had to perform most of the experiment. However, a member of the team periodically assisted or observed them.

Materials
The team's lab was not equipped to carry out this experiment. As a result, Dr. Jenne kindly offered us to work alongside with members of his team in his lab. All the materials were provided by Dr. Jenne.

- Formalin
- Optimal Cutting Temperature (OCT)
- Cryomold
- Blade (VWR Cat #: 95057-832, coated microtone blades)
- Cryostat
- Staining Glass Container and Acetone
- 2% BSA+PBS
- Fluorescent mounting medium (Dako, S3023)

- Conjugated Antibody:
    - Biolegend FITC anti-mouse Ly6G (clone 1A8), cat#:127606.
    - BD Pharmingen PE anti-mouse CD49b (clone HMalpha), cat#:558759.
    - eBioscience APC anti-mouse CD45 (clone 30-F11), cat#:17-0451-83.
- Fluorescent Microscope

Protocol

1. Collect the tissue samples from the lumbar region of the mouse after euthanization.
2. Tissue Fixation:
    - Formalin:
        - Remove the wax that might come from Nair. The mice skin tissue sample might contain some wax (from Nair during transdermal patch application).
        - Treat the tissue with the formalin solution to fix it.
        - The fixed tissue can then be stored at -80 ^{0}C.

3.. Cryosection:

i) Fill an ice box with dry ice, some ethanol, and a piece of metal.

ii) Place some Optimal Cutting Temperature (OCT) compound and the tissue in a cryo mold. The orientation of the tissue should be such that all the layers of the tissue are obtained.

iii) Fill cryo mold with OCT compound to cover the whole tissue (approximately 0.5 cm from top of the mold).

iv)When the samples are completely frozen, wrap individual samples in labelled foils, seal in plastic bag, and store in -80°C..

v) Put blade (VWR Cat #: 95057-832, coated microtone blades) in the Cryostat machine and tighten.

vi) Anchor tissue sample and adjust it so it's parallel to the blade.

vii) Prime tissue sample.

viii) Slowly start cutting .

ix) Allow the section to dry,approximately 3 hrs.

x) Blade was discarded.

4. Cryosection FIxation:

i) Insert tissue slide in staining glass container that contains Acetone.

ii) Incubate at room temperature for 10 mins.

iii) Fill the staining glass container containing the slides with prepared 2% BSA+PBS.

iv) Incubate at room temperature for 2 hours.

v) Wipe the slide in such a way that a dome shape is created, and add 2% BSA+PBS to the dome.

vi) Add 1 uL of conjugated antibody to the dome. Incubate at room temperature for 1 hr. Add some water to keep the slide moist at all times.

- Biolegend FITC anti-mouse Ly6G (clone 1A8), cat#:127606
- BD Pharmingen PE anti-mouse CD49b (clone HMalpha), cat#:558759

- eBioscience APC anti-mouse CD45 (clone 30-F11), cat#:17-0451-83

vii) Wash the slide three times with 2% BSA+PBS.

viii) Add 1 drop of Fluorescent mounting medium (Dako, S3023) to slide.

ix) Gently load the cover slip.

x) Keep tissue slide at 4C.

5.. Sample Visualization through fluorescent microscopy.

Results

The control tissue was not stained properly, and as such the image of the section holds no scientific value. Despite the failure of staining in control tissue samples, behavioural conclusions can still be made. No unusual behaviour was observed in mice after the application of the patch. Furthermore, no rashes or any skin problems were observed on the mice skin.

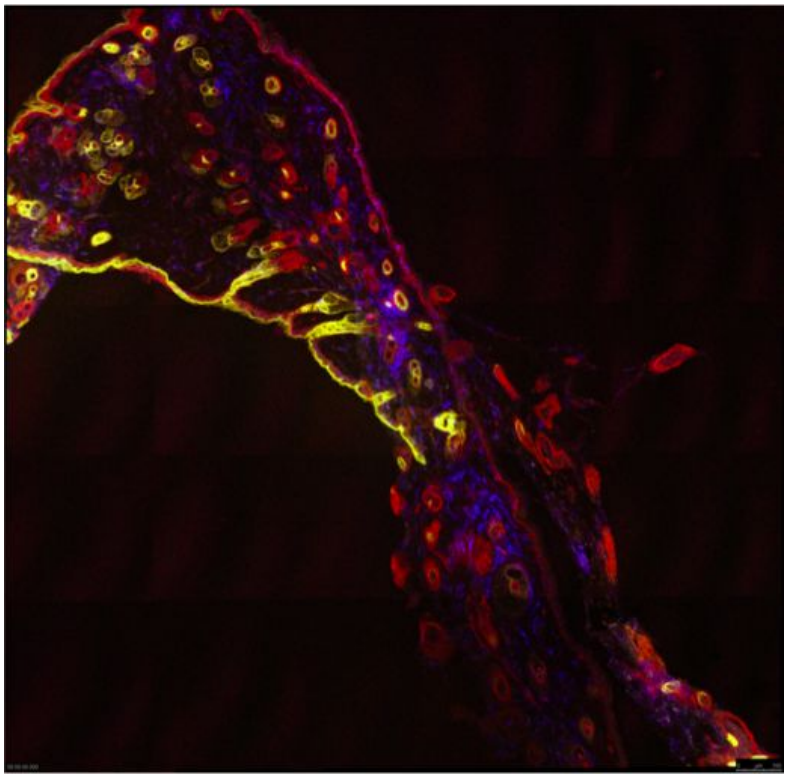


Figure 1: The chassis treatment mice skin tissue (day 5, *Bacillus subtilis*) was subjected to Immunohistochemical staining. The sample was then visualized by fluorescent microscopy. The blue color corresponds to APC anti-mouse CD45 antibody (lymphocytes), the green color corresponds to FITC anti-mouse Ly6G antibody (neutrophils), and the red color corresponds to Pharmingen PE anti-mouse CD49b antibody (platelets). The yellow color is a combination of the red color and green color.

Future Experiments:

In the future, we would like to perform immunohistological staining with all the treatments of skin tissue samples. Further steps would to be taken to ensure that patch adheres to the skin for the entirety of the treatment. As well, for more statistical significance, study needs to be carried out with more mice per treatment.